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ATTORNEY'S DOCKET NUMBER	
06478.1461	
U.S APPLICATION NO (If known, see 37 CFR 1.5)	10/018815

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/04101	June 22, 2000	June 23, 1999

TITLE OF INVENTION
HUMAN ANTITHROMBIN VARIANTS

APPLICANT(S) FOR DO/EO/US
Takehiko KOIDE

Applicant(s) herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c)(2)).
   a. ☐ is attached hereto (required only if not communicated by the International Bureau
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed with the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154 (d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3)).
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☒ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ Information Disclosure Statement under 37 CFR 1.97 and 1.98
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3 28 and 3.31 is included.
13. ☐ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A Substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154 (d)(4).
19. ☐ A second copy of the English language translation of the international application 35 U.S.C 154 (d)(4).
20. ☒ Other items or information:
   a. ☒ Copy of cover page of International Publication No. WO 00/78811
   b. ☐ Copy of Notification of Missing Requirements.
   c. ☒ Copy of International Search Report

[redacted] RECEIVED - 21 DEC 2001

U.S. APPLICATION NO (If known, see 37CFR 1.5)	APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/018815	PCT/JP00/04101	06478.1461

21. ☒ The following fees are submitted:

CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTOand all claims satisfied provisions of PCT Article 33 (1)-(4) **$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =				$890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				$	
CLAIMS	NUMBER FILED		NUMBER EXTRA	RATE	
Total Claims	6	- 20 =		x $18.00	$
Independent Claims	1	-3 =		x $84.00	$
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+$280.00	$
TOTAL OF THE ABOVE CALCULATIONS =				$890.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½				$	
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Fee for recording the enclosed assignment (37 CFR 1.21 (h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property. +				$	
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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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DATED: December 21, 2001

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[INTERNATIONAL APPLICATION NO.] PCT/JP00/04101
[INTERNATIONAL PUBLICATION NO.] WO 00/78811 A1

SPECIFICATION

Human Antithrombin Variants

[TECHNICAL FIELD TO WHICH THE INVENTION BELONGS]

This invention relates to an artificial human antithrombin variant which has a high protease inhibitory activity in the absence of heparin. More particularly, the invention relates to a human antithrombin variant having a three dimensional structure after binding to heparin, wherein the three dimensional structure of natural human antithrombin molecule is modified by a genetic engineering procedure. The variant of the invention can be used for the treatment of, for example, DIC, thrombotic diseases or gestosis.

[PRIOR ART]

It has been disclosed that there are various kinds of antithrombin activities in natural antithrombin, and antithrombins I to VI have been proposed. However, in view of the fact that only antithrombin III has been isolated as protein up to the present time, antithrombin III is now referred to simply as antithrombin. Accordingly, the antithrombin III is hereinafter referred to as antithrombin in regard to this invention.

Natural human antithrombin is a single-strand glycoprotein having a molecular weight of 58,000, which has an inhibitory activity on proteases in the blood

coagulation system. Natural human antithrombin is biosynthesized as a precursor protein consisting of 464 amino acid residues, but a signal peptide consisting of 32 residues is cleaved out in the course of secretion. Thus, mature human antithrombin circulating in blood vessels is composed of 432 amino acid residues. All of six cysteine residues (Cys) form disulfide bonds, and human antithrombin molecule is stabilized with the S-S bridges at three sites of Cys8-Cys128, Cys21-Cys95 and Cys247-Cys430. Natural human antithrombin contains approximately 15% of sugars, and complex-type sugar chains are attached to asparagine residues at four positions (Asn96, Asn135, Asn155 and Asn192). The molecular site of natural human antithrombin which interacts directly with and binds to the active center of protease is referred to as a reactive site, which is Arg393-Ser394 located near the C-terminus of peptide chain.

Natural human antithrombin is a plasma protein belonging to a serpin superfamily like α_1-antitrypsin and heparin cofactor II, and is a main control factor in the blood coagulation system which controls activities of main coagulating enzymes, such as thrombin, activated factor X (factor Xa), activated factor IX (factor IXa), etc. Natural human antithrombin having such pharmacological activities has been used for the normalization of abnormally accelerated coagulation, more specifically, disseminated intravascular coagulation (DIC), and gestosis whose main symptoms are hypertension, proteinuria and edema during the period of

pregnancy, as well as for treating hyper-thrombopoiesis derived from congenital human antithrombin deficiency.

It is well known that natural human antithrombin has a high affinity for heparin and that inhibition rates on thrombin and factor Xa can be accelerated to 1000 times and 300 times, respectively, in the presence of heparin.

Analyses of primary structure level so far made have revealed that a heparin-binding site is located in the N-terminal region of natural human antithrombin and the reactive site with protease is located near the C-terminus (Arg393-Ser394). Moreover, 5-10% of natural human antithrombin in blood is a molecular species wherein a sugar chain is not bound to Asn135 (human antithrombin β), and this species reveals a higher heparin affinity than a dominant molecular species (human antithrombin α) does.

Natural human antithrombin molecule is, like other blood serpins, a protein which comprises, in a three-dimensional structure, several strands (hereinafter abbreviated as s1A - s4C) composed of antiparallel β sheets, roughly classified to three directions of A, B and C, nine α-helixes (hereinafter abbreviated as hA - hI) and a coil structure moiety (Stein PE, Carrell RW, Nature Struct Biol 2: 96, 1995). Recently, reports on X-ray crystal structure at 2.6 Å of the antithrombin dimers of native form and latent form (Skinner R., et al., J. Mol. Biol. 266: 601, 1997) and on crystal structures at 2.9 Å of a complex with pentasaccharide of the core moiety of a high-affinity heparin (Jin L., et al., Proc. Natl. Acad. Sci. USA, Vol. 94: 14683, 1997)

have revealed a three-dimensional interacting site of natural human antithrombin with heparin and dynamic structural change in antithrombin molecule caused by heparin binding.

On the basis of these dynamic structural changes in natural human antithrombin molecule, the present inventor has thought that human antithrombin is an "incomplete" serpin as an inhibitor in the absence of heparin and would become a "complete" inhibitor only in the presence of heparin.

On the basis of these prior findings, preparation of a human antithrombin variant having a high protease-inhibitory activity even in the absence of heparin has been attempted by exchanging amino acid(s) at the specific position(s) in natural human antithrombin. For example, a human antithrombin variant is disclosed wherein one, two or more of the amino acids at positions 49, 96, 135, 155, 192, 393 and 394 in natural human antithrombin are replaced with other amino acids (Japanese Patent Kokai No. 262598/1990). Also, another human antithrombin variant is disclosed wherein at least one of the amino acids in four regions of positions 11 - 14, 41 - 47, 125 - 133 and 384 - 398 is replaced with other amino acids, alone or in combination in the respective regions (Japanese Patent Kokai No. 339292/1993). Since these variants do not always exert a satisfactory effect, there has been a demand for the preparation of a human antithrombin variant having a still more potent protease-inhibitory activity in the absence of heparin.

[DISCLOSURE OF THE INVENTION]

Purpose of the present invention is to provide a novel human antithrombin variant, which can act as a complete inhibitor in the absence of heparin and exert a high protease-inhibitory activity.

When natural human antithrombin acts as a protease inhibitor, a great conformational change occurs in the reactive loop of human antithrombin. More specifically, the reactive loop protruding from the molecule surface of natural human antithrombin is recognized as a "substrate" for target protease, and the peptide bond at the reactive site [P1 (Arg393)-P1' (Ser394)] is cleaved with the protease. At this time, an acyl bond is formed between the carbon of the carbonyl group of Arg393 at P1 and the oxygen of the hydroxyl group of the active center Ser195 of the protease, whereby an acyl-enzyme complex is formed and, simultaneously, 15 residues (P1 - P15) on the N-terminus of the cleaved reactive loop are incorporated between s3A and s5A to form a new strand (s4A). At this point, Arg393 moves approximately 70 Å from one end to another in human antithrombin molecule, together with the protease. This dynamic change is thought to be significant to form a stable complex with a protease. There has also been found a latent form of natural human antithrombin or plasminogen activator inhibitor 1 wherein the reactive site, though not cleaved, is inserted into the molecule as s4A. From these facts, a stable structure of serpin is thought to reside in the formation of s4A.

Since the reactive loop of natural α-antitrypsin is completely exposed over the molecule surface and the side chain of Met358 at P1 is oriented outwardly from

the molecule to form complementary conformation to the active site of serine protease, reactivity with protease is high and intramolecular insertion of the reactive loop after cleavage is apt to occur. However, since the side chain of Arg393 at P1 of natural human antithrombin is oriented inwardly in the molecule, reactivity with protease is extremely low (Jin L., et al., Proc. Natl. Acad. Sci. USA, 94: 14683, 1997). The present inventor's attention has been drawn to a still more significant fact that the reactive loop in natural human antithrombin is incorporated into strands at P14 (Ser380) and P15 (Gly379), which provides strands with distortion and also makes insertion of the cleaved loop difficult. Binding of heparin to natural human antithrombin induces conformational changes at various sites of human antithrombin molecule, and the amino acids at P14 (Ser380) and P15 (Gly379) could be extruded from the strand by an allosteric effect (steric hindrance effect) of heparin binding and dislocated to the same location as in α-antitrypsin (Jin L., et al., Proc. Natl. Acad. Sci. USA, 94: 14683, 1997).

The present inventor has analyzed and studied conformational change in the respective reactive loops of the above human antithrombin, plasminogen activator inhibitor 1 and α_1-antitrypsin, and, as a result, have judged that P14 (Ser380) in natural human antithrombin is a key site for constructing a suitable three dimensional structure acting as a complete inhibitor in the absence of heparin and having a high protease-inhibitory activity. Moreover, in view of that the site of P15 - P10 in the reactive loop of natural human

antithrombin is referred to as "proximal hinge" region, the present inventor has studied its three dimensional structural feature in human antithrombin and have found that this proximal hinge region plays a role as a hinge when the reactive loop is incorporated as s4A. Based on the finding, the present inventor has judged that the site at P16 (Glu378) corresponding to the base of said hinge is also a site to be replaced by another suitable amino acid for the preparation of human antithrombin variant having a proper three dimensional structure and a high protease-inhibitory activity, together with the site at P14.

On the other hand, it is known that the reactive loop in natural human antithrombin is inserted into the molecule as s4A between s3A and s5A when cleaved with a protease and that the region participating in opening these strands is the shutter region wherein hB (Ser79 - Thr90) is centered (Stein PE, Carrel RW, Nature Struct Biol 2: 96, 1995). The present inventor has found that, on opening between s3A and s5A, the hydrogen bonds between both strands are first cleaved and then these strands slide over the groove of hB, and that "easier opening" of this region relates to "easier inserting" of the reactive loops. In summary, the shutter region in natural human antithrombin is an important region which influences upon the opening and closing between s3A and s5A and further influences upon binding of heparin and activity of antithrombin. Then, the present inventor has judged that a human antithrombin variant having a three dimensional structure with a high protease-inhibitory activity can be produced by replacing the

amino acid at position 78 (Leu78), which corresponds to the base of the shutter region, with another amino acid.

Then, the present inventor has analyzed and studied dynamic structural changes in natural human antithrombin and promotion of protease-inhibitory activity, which are induced by heparin binding; in particular, the three dimensional structure of each amino acid in the heparin binding region. It has been hitherto elucidated that the heparin binding region in natural human antithrombin consists of a group of basic amino acid residues which are located at hA and hD, based on analysis of anomalous cases such as antithrombin TOYAMA wherein Arg at position 47 is replaced with Cys (Koide T., Takahashi K., et al., Proc. Natl. Acad. Sci. USA, 81: 289, 1984) or chemical modification experiments, as well as analysis of variants prepared by site-specific mutagenesis. The above-mentioned X-ray analysis of crystal structure (Jin L., et al., Proc. Natl. Acad. Sci. USA, 94: 14683, 1997) has elucidated that the heparin-derived pentasaccharide binding sites in natural human antithrombin are in hD (side chains of Lys125 and Arg129), hA (side chains of Arg46 and Arg47, and main chain amide of Asn45), the N-terminal region (side chains and main chain amides of Lys11 and Arg13), and main chain amide of Glu113 and side chain and main chain amides of Lys114 in the "P-helix" (P is originated from pentasaccharide), which is formed between hC-hD through binding with pentasaccharide. When pentasaccharide comes into contact, Arg46 and Arg47 move by 17 Å and 8 Å, respectively, to form a hydrogen bond with the sulfate

group of the sugar chain. Moreover, hD is inclined by an angle of about 10 degrees toward the direction of pushing s2A and s3A, and the coil structure of Glu113 - Gln118 on its N-terminal side is modified to two-twisted hP toward the right-angled direction to hD. Moreover, a one and half-twisted helix is also formed on the C-terminal side of hD, in such ways that side chains of Arg132, Lys133 and Lys136 are directed toward the pentasaccharide binding site. These residues are far apart from the pentsaccharides and thus a hydrogen bond is not formed between them, but it is highly possible for these residues to interact with a long-chain heparin. Moreover, in the said native form of human antithrombin, the amino acid residues at P14 and P15 in the hindered reactive loop is extruded by an allosteric effect caused by elongation of hD, and distortion of strands is eliminated and simultaneously the side chain of Arg393 at P1 is directed outwardly from the molecule, which transforms into a form which may react as an inhibitor (Pike RN, et al., J. Biol. Chem. 272: 19652, 1997). Moreover, the N-terminal region of human antithrombin (Ile22 - Arg46) moves greatly when bound with pentasaccharide and thus plays a role as a steric gate for stabilizing an antithrombin-pentasaccharide complex (Fittom HL., et al., Protein Science 7: 782, 1998). In comparing three dimensional structures of the native form and latent form of natural human antithrombin, hD of the native form is slightly twisted, heparin binding site, Arg47, Lys125 and Arg129, are directed toward the pentasaccharide binding region, and the Nε group of Arg129 forms a hydrogen bond with the

side chain of Asp278 to stabilize the said side chain, which facilitates ionic interaction with the sulfate groups of pentasaccharide. However, in the latent form, hD elongates straightforward, Arg47 and Lys125 form hydrogen bonds with Ser112 and Ile7, respectively, and all amino acid residue regions, which are significant for heparin binding, are not directed toward heparin binding regions (Skinner R., et al., J. Mol. Biol. 266: 601, 1997). In view of this, the present inventor has judged that the three dimensional structure may be altered, even in the absence of heparin, to a conformation similar to that in the presence of heparin, by prior cleavage of a hydrogen bond between Arg129 and Asp278. Based on the judgement, the present inventor thought that replacing the amino acid at position 278 (Asp278), which binds to Arg129 via a hydrogen bond, with another amino acid can produce an antithrombin variant having a suitable three dimensional structure with a high protease-inhibitory activity.

As depicted above, the present inventor has studied information on dynamic conformational change of antithrombin resulting from binding of heparin to natural human antithrombin as previously analyzed and, as a result, have found out a preferred site to be modified or altered in the three dimensional structure for promoting protease-inhibitory activity of natural human antithrombin. More specifically, the present inventor has reached the conclusion that replacement with one, two or more of other amino acids in the hinge region of the reactive loop of natural human antithrombin, the hinge region to form s4A, as well as

those sites involved in heparin binding can produce a human antithrombin variant having a suitable three dimensional structure with a high protease-inhibitory activity. The present inventor has made the earnest studies on improvement of human antithrombin variant based on the above-mentioned conclusion, and finally succeeded in preparing a novel human antithrombin variant having an appropriate three dimensional structure for a high protease-inhibitory activity, upon which this invention has been completed.

The present invention is directed to a human antithrombin variant, that is, a human antithrombin variant wherein at least one of amino acids at positions 78, 278, 378 and 380 in the amino acid sequence of natural human antithrombin is replaced with another amino acid. Of these human antithrombin variants, the following variants are particularly preferred:

a human antithrombin variant wherein the amino acid at position 78 in the amino acid sequence of natural human antithrombin is replaced with Phe,

a human antithrombin variant wherein the amino acid at position 278 in the amino acid sequence of natural human antithrombin is replaced with the amino acid selected from Ala, Arg, Asn, Gly, His, Tyr and Val,

a human antithrombin variant wherein the amino acid at position 378 in the amino acid sequence of natural human antithrombin is replaced with the amino acid selected from Lys, Asn and Val, and

a human antithrombin variant wherein the amino acid at position 380 in the amino acid sequence of natural human antithrombin is replaced with the amino

acid selected from Ala, Asp, Gly, His, Ile, Leu, Asn, Pro, Arg, Thr, Tyr and Val.

Moreover, the invention is directed to a DNA encoding the said human antithrombin variant.

[BRIEF DESCRIPTION OF DRAWING]

Figure 1 shows construction of an expression vector for an antithrombin (AT) recombinant variant (in case of Ser380His).

[DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS]

The novel antithrombin variant of the invention was prepared by site-specific mutagenesis as a variant having a three dimensional structure, which is similar to that after binding with heparin.

The invention is more specifically illustrated by way of the specific method for the preparation of a variant as set forth hereinafter.

To 2.5 μg (10 μl) of cDNA (single-stranded) for the native form of antithrombin was annealed 30 μl of a variant primer (0.475 OD/ml) for amino acid substitution to synthesize a full-length cDNA with DNA polymerase. Then, the nucleotide sequence was determined to confirm a formation of variant. cDNA (1.4kb) for each antithrombin variant was integrated into EcoRl site of pcD2 vector and then cleaved with EcoRI and PstI to confirm the direction of the inserted sequence. The vector with sequence integrated in right direction was transfected into BHK cells for a large-scale production by a calcium phosphate method (FIG. 1). The neomycin-resistant, stably expressing cells were selected with G418 and pooled. The pooled stably expressing BHK (baby hamster kidney) cells were used to conduct pulse-chase

experiment. $5x10^5$ cells were seeded onto a 35 mm dish in diameter and cultured overnight. They were labeled with 6.8 µl of EXRE35S35S (100 µCi/ml) for 30 minutes. Then the cultured broth was exchanged with DME/10%FCS, Met, Cys, and then chase was performed for 8 hours to obtain culture media (CM) and cell extracts (CE) after 0, 0.5, 1, 2, 4 and 8 hours. The CM and CE at each point were immune-precipitated with an antibody and Staphylosorb™, then 8% SDS-PAGE (+SH) was performed, and the radioactivity in the resulting RI bands was measured to determine the secreted amounts of the recombinant antithrombin variant.

For the cell secreting a high amount of the antithrombin variant, the CM was collected after 8 hours chase. To 500 µl of the collected liquid was added thrombin or factor Xa, and, after reacting at 37°C for 5 minutes or 60 minutes without heparin, or for 5 minutes with heparin, an immune precipitation was performed and an amount of the complex was determined by 10% SDS-PAGE (+SH).

1) Secretion

Secretion from BHK cells of each recombinant antithrombin variant is summarized in Table 1, which shows intracellular and secreted amounts after 8 hours chase, taking the radioactivity in pulse-labeling as 100%. A secreted amount of the native-type recombinant antithrombin was 89%, whereas Leu78Phe variant wherein Leu at position 78 was replaced with Phe showed a secretion amount of 90%. The variant wherein Asp at position 278 was replaced with Ala, Gly, His or Tyr (Asp278Ala, Asp278Gly, Asp278His or Asp278Tyr) showed a

secretion of 104%, 104%, 165% or 160%, respectively, which were higher than that of the native-type recombinant antithrombin.

On the other hand, the variant wherein Asp at position 278 was replaced with Arg, Asn or Val (Asp278Arg, Asp278Asn or Asp278Val) showed secretion of 57%, 48% or 51%, respectively. A satisfactory secretion was given in all variants wherein Ser at position 380 was replaced with Ala, Arg, Asn, Asp, Gly, His, Pro, Thr, Tyr or Val (Ser380Ala, Ser380Arg, Ser380Asn, Ser380Asp, Ser380Gly, Ser380His, Ser380Pro, Ser380Thr, Ser380Tyr or Ser380Val). Especially, the variants replaced with Asn and Val provided a high secretion of 154% and 144%, respectively.

2) Complex-forming ability with thrombin (TAT)

The results of studies on thrombin-antithrombin complex (TAT)-forming ability of each recombinant antithrombin variant are summarized in Table 2. Immediate TAT-forming ability in the absence of heparin, which is the greatest effect of the invention, was 131% for the Leu78Phe variant wherein Leu at position 78 was replaced with Phe, 163% for the Asp278His variant wherein Asp at position 278 was replaced with His, and 171% and 172% for the Ser380Gly and Ser380Tyr variants wherein Ser at position 380 was replaced with Gly and Tyr, respectively, in terms of the relative value when TAT-forming ability of a native type of recombinant is defined as 100%, and variants having a higher efficacy than that of a native type of recombinant were provided in every case. Moreover as shown in Table 2, in the interaction with thrombin over a prolonged period of

time (120 minutes), Leu78Phe, Asp278His and Ser380Ala variants had the same level of a stable TAT-forming ability as the native type of recombinant antithrombin, and Asp278Ala, Asp278Val, Asp278Tyr, Ser380Gly and Ser380Tyr variants had a higher level of a stable TAT-forming ability than the native type of recombinant antithrombin.

Moreover, all variants could retain the immediate TAT-forming ability in the presence of heparin and have also been demonstrated as an effective antithrombotic agent to be used in combination with heparin.

3) Complex-forming ability with factor Xa (Xa-AT)

The results of studies on factor Xa-antithrombin complex (Xa-AT)-forming ability of each recombinant antithrombin variant with factor Xa (Xa-AT) are summarized in Table 3. Immediate Xa-AT-forming ability in the absence of heparin, which is the greatest effect of this invention, was 106% for the Leu78Phe variant wherein Leu at position 78 was replaced with Phe, in terms of the relative value when Xa-AT-forming ability of a native-type of recombinant is defined as 100%. Also, values of 144%, 171% and 131% were obtained in the cases of Asp278Gly, Asp278His and Asp278Tyr variants wherein Asp at position 278 was replaced with Gly, His, or Tyr, respectively, and there could be obtained those variants having a higher efficacy than a native-type of recombinant antithrombin in every case. Moreover, in the interaction with factor Xa over a prolonged period of time (60 minutes), Leu78Phe, Asp278Gly, Asp278His and Ser380Tyr variants had the same level of a stable TAT-forming ability as the native-type of recombinant, and

Asp278Val, Asp278Tyr and Ser380Gly variants had a higher level of a stable TAT-forming ability than the native-type of recombinant antithrombin.

Moreover, the immediate Xa-AT-forming ability in the presence of heparin in the cases of Leu78Phe, Asp278Ala and Asp278Gly variants was decreased approximately by half, as compared with native-type of recombinant antithrombin, and this has demonstrated effectiveness of these variants as a non-heparin-dependent inhibitor for factor Xa with a high efficacy. On the other hand, the Asp278Val, Asp278Tyr, Ser380Gly, Ser380Thr and Ser380Tyr variants have retained the immediate TAT-forming ability in the presence of heparin, thereby demonstrating their effectiveness as an antithrombotic agent to be used in combination with heparin.

Table 1 □□Secretion of AT Recombinant Variant

Intracellular and secreted amounts after 8 hours chase, taking the radioactivity in pulse-labeling as 100%

Recombinant	Intracellular amount (%)	Secreted amount (%)	Total
Native	1.4	89	90.4
Leu78Phe	10	90	100
Asp278Ala	16	104	120
AsP278Arg	4.6	57	61.6
Asp278Asn	4.9	48	52.9
AsP278Gly	22	104	126
AsP278His	9.2	165	174.2
Asp278Tyr	16	160	176
AsP278Val	4.6	51	55.6
Glu378Lys	15	62	77
Ser380Ala	4.9	79	83.9
Ser380Arg	10	73	83
Ser380Asn	38	154	192
Ser380Asp	10	83	93
Ser380Gly	6.1	128	134.1
Ser380His	8.3	120	128.3
Ser380Pro	30	63	93
Ser380Thr	13	122	135
Ser380Tyr	11	78	89
Ser380Val	17	144	161

Table 2 TAT-Complex Forming by AT Recombinant Variant

Recombinant AT	TAT (%) (-) heparin, 5 min.	TAT (%) (-) heparin, 120 min.	TAT (%) (+) heparin, 5 min.
Native	100	100	100
Leu78Phe	131	93	81
Asp278Ala	102	108	99
Asp278His	163	95	89
Asp278Val	90	108	104
Asp278Tyr	105	106	104
Ser380Ala	56	86	118
Ser380Gly	171	112	98
Ser380Tyr	172	122	111

Value expressed in terms of the relative value when TAT-forming ability of native-type of recombinant AT is defined as 100%

Table 3 Xa-AT-Complex Forming by AT Recombinant Variant

Recombinant AT	Xa-AT (%) (-) heparin, 5 min.	Xa-AT (%) (-) heparin, 120 min.	Xa-AT (%) (+) heparin, 5 min.
Native	100	100	100
Leu78Phe	106	88	53
Asp278Ala	80	56	44

Asp278Gly	144	87	54
Asp278His	171	80	89
Asp278Val	89	116	136
Asp278Tyr	131	156	161
Ser380Gly	56	114	168
Ser380Thr	8.8	52	128
Ser380Tyr	86	90	105

Value expressed in terms of the relative value when Xa-AT-forming ability of native-type of recombinant AT is defined as 100%

[INDUSTRIAL UTILITY OF THE INVENTION]

According to the present invention, there can be provided a novel human antithrombin variant, which has an appropriate three dimensional structure capable of exhibiting a high protease-inhibitory activity even if heparin is not present. The recombinant human antithrombin variant of the invention is useful as a therapeutic agent of, for example, thrombotic diseases or gestosis.

[CLAIM]

1. A human antithrombin variant characterized in that at least one of the amino acids at positions 78, 278, 378 and 380 in the amino acid sequence of natural human antithrombin is replaced with another amino acid.

2. The human antithrombin variant as claimed in claim 1 wherein the amino acid at position 78 in the amino acid sequence of natural human antithrombin is replaced with Phe.

3. The human antithrombin variant as claimed in claim 1 wherein the amino acid at position 278 in the amino acid sequence of natural human antithrombin is replaced with the amino acid selected from Ala, Arg, Asn, Gly, His, Tyr and Val.

4. The human antithrombin variant as claimed in claim 1 wherein the amino acid at position 378 in the amino acid sequence of natural human antithrombin is replaced with the amino acid selected from Lys, Asn and Val.

5. The human antithrombin variant as claimed in claim 1 wherein the amino acid at position 380 in the amino acid sequence of natural human antithrombin is replaced with the amino acid selected from Ala, Asp, Gly, His, Ile, Leu, Asn, Pro, Arg, Thr, Tyr and Val.

6. A DNA encoding the human antithrombin variant as claimed in claim 1.

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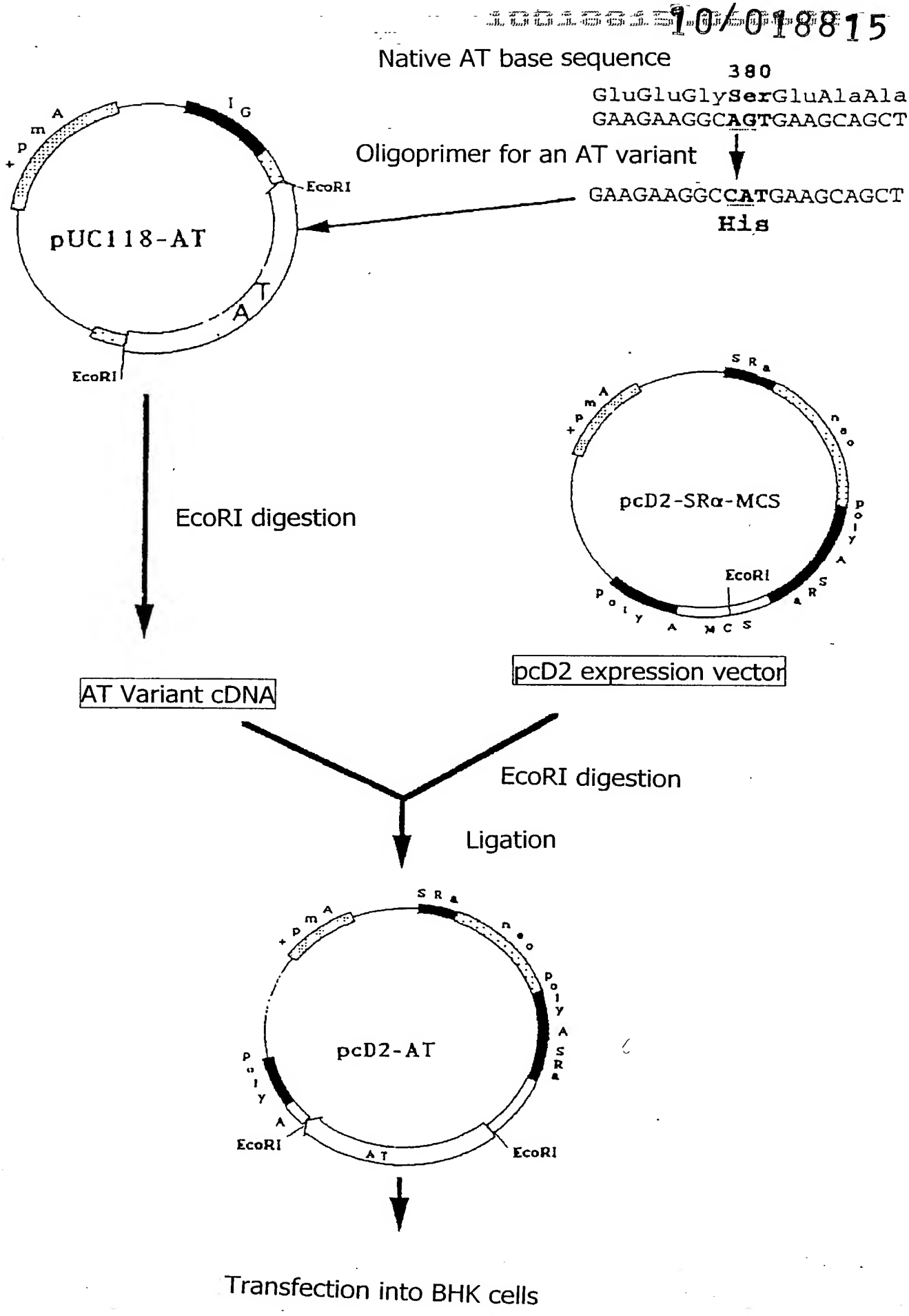
[TITLE] Human Antithrombin Variants

[ABSTRACT]
Human antithrombin variants showing a high protease inhibitory activity even in the absence of heparin wherein at least one of the amino acids at positions 78, 278, 378 and 380 in the amino acid sequence of natural human antithrombin is substituted by another amino acid. Preferable examples thereof are human antithrombin variants wherein the amino acid at position 78 is substituted by Phe; the amino acid at position 278 is substituted by Ala, Arg, Asn, Gly, His, Tyr or Val; the amino acid at position 378 is substituted by Lys, Asn or Val; and/or the amino acid at position 380 is substituted by Ala, Asp, Gly, His, Ile, Leu, Asn, Pro, Arg, Thr, Tyr or Val.


Native AT base sequence
380
GluGluGlySerGluAlaAla
GAAGAAGGCAGTGAAGCAGCT
Oligoprimer for an AT variant
GAAGAAGGCCATGAAGCAGCT
His
pUC118-AT
EcoRI
EcoRI
EcoRI digestion
pcD2-SRα-MCS
EcoRI
pcD2 expression vector
AT Variant cDNA
EcoRI digestion
Ligation
pcD2-AT
EcoRI
EcoRI
Transfection into BHK cells

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

HUMAN ANTITHROMBIN VARIANTS

the specification of which
☐ is attached and/or
☒ was filed on December 21, 2001 as United States Application Serial No. 10/018,815 or
☒ on June 22, 2000, as PCT International Application No. PCT/JP00/04101and was amended on April 6, 2001.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate or § 365(a) of any PCT international application(s) designating at least one country other than the United States, listed below and have also identified below, any foreign application(s) for patent or inventor's certificate, or any PCT International application(s) having a filing date before that of the application(s) of which priority is claimed:

Country	Application Number	Date of Filing	Priority Claimed Under 35 U.S.C. 119
Japan	11-176967	June 23, 1999	☒ YES ☐ NO
			☐ YES ☐ NO

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Number	Date of Filing

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) or § 365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application(s) in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application(s) and the national or PCT International filing date of this application:

Application Number	Date of Filing	Status (Patented, Pending, Abandoned)

I hereby appoint the following attorney and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. **FINNEGAN, HENDERSON, FARABOW, GARRETT & DUNNER, L.L.P., CUSTOMER NUMBER 22,852,** Douglas B. Henderson, Reg. No. 20,291; Ford F. Farabow, Jr., Reg. No. 20,630; Arthur S. Garrett, Reg. No. 20,338; Donald R. Dunner, Reg. No. 19,073; Brian G. Brunsvold, Reg. No. 22,593; Tipton D. Jennings, IV, Reg. No. 20,645; Jerry D. Voight, Reg. No. 23,020; Laurence R. Hefter, Reg. No. 20,827; Kenneth E. Payne, Reg. No. 23,098; Herbert H. Mintz, Reg. No. 26,691; C. Larry O'Rourke, Reg. No. 26,014; Albert J. Santorelli, Reg. No. 22,610; Michael C. Elmer, Reg. No. 25,857; Richard H. Smith, Reg. No. 20,609; Stephen L. Peterson, Reg. No. 26,325; John M. Romary, Reg. No. 26,331; Bruce C. Zotter, Reg. No. 27,680; Dennis P. O'Reilley, Reg. No. 27,932; Allen M. Sokal, Reg. No. 26,695; Robert D. Bajefsky, Reg. No. 25,387; Richard L. Stroup, Reg. No. 28,478; David W. Hill, Reg. No. 28,220; Thomas L. Irving, Reg. No. 28,619; Charles E. Lipsey, Reg. No. 28,165; Thomas W. Winland, Reg. No. 27,605; Basil J. Lewris, Reg. No. 28,818; Martin I. Fuchs, Reg. No. 28,508; E. Robert Yoches, Reg. No. 30,120; Barry W. Graham, Reg. No. 29,924; Susan Haberman Griffen, Reg. No. 30,907; Richard B. Racine, Reg. No. 30,415; Thomas H. Jenkins, Reg. No. 30,857; Robert E. Converse, Jr., Reg. No. 27,432; Clair X. Mullen, Jr., Reg. No. 20,348; Christopher P. Foley, Reg. No. 31,354; John C. Paul, Reg. No. 30,413; Roger D. Taylor, Reg. No. 28,992; David M. Kelly, Reg. No. 30,953; Kenneth J. Meyers, Reg. No. 25,146; Carol P. Einaudi, Reg. No. 32,220;

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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